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Evaluation of Fcγ receptor mediated T-cell activation by two purified CD3 × CD19 bispecific monoclonal antibodies with hybrid Fc domains

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Summary Two bispecific monoclonal antibodies (BsAb), differing in H chain isotype combination, were made for treatment of B-cell leukaemia/lymphoma; QAI-2, CD3-mouse-IgG_1 × CD19-mouse-IgG_2a and QAI-3, CD3-mouse-IgG_1 × CD19-mouse-IgG_2b. Both purified BsAb proved equally effective for their ability to target pre-activated T cells towards CD19 positive tumour cells. In T-cell proliferation assays, the capacity of FcγRIa (CD64), FcγRIIa-R131 and FcγRIIa-H131 (CD32) transfected fibroblasts was tested to present the BsAb. The BsAb combining mouse (m) IgG_1 and $mIgG_2a$ promoted T-cell activation in combination with the FcγRIa transfectant; the $mIgG_1$–$mIgG_2b$ BsAb was only marginally active. Both BsAb could not induce T-cell activation when presented by either of the FcγRIIa transfectants. Similar results were obtained using PBMC cultures, containing $Fc\gamma RIa^+/Fc\gamma RIIa^+$ monocytes as accessory cells. The importance of FcγR-dependent BsAb-mediated T-cell activation emerged from experiments with T cells and CD19 positive B-cell lines, showing that cross-linking via $CD19^+$ target cells alone did not induce T-cell proliferation. Therefore, BsAb with functionally different Fc domains represent alternative strategies in BsAb therapy, the efficacy of which deserves to be compared in vivo.

Keywords bispecific hybrid mAb; cancer therapy; Fcγ receptor transfectants; mouse isotypes; T-cell activation.

Introduction

Bispecific monoclonal antibody (BsAb) therapy may be performed with biologically produced intact Ig molecules (1). The H-chain isotype composition will, thereby, influence the ability of the BsAb to interact with Fcγ receptor positive cells. Such Ab interactions might lead to negative side effects as a result of (increased) cytokine release (2,3). These FcγR interactions, however, can also result in favourable T-cell activation (4–6) which may even be a prerequisite for efficient killing of tumour cells upon targeting by the BsAb.

For the treatment of patients with B lineage leukaemia/lymphoma we intend to use BsAb with specificities for the B-cell marker CD19 and the T-cell complex CD3. To be able to compare positive and negative contributions of FcγR interactions with BsAb *in vitro* and *in vivo*, we constructed two hybrid hybridomas, each producing a BsAb with identical binding sites for the CD3 and CD19 antigen, but differing in isotype composition of their Fc domain. The first BsAb was derived from mouse (m) IgG_1 and $mIgG_2a$ mAb, while in the second BsAb the $mIgG_2a$ was replaced by the $mIgG_2b$ isotype (Table 1). Human monocytes express FcγRIa (CD64) and FcγRIIa (CD32) (7). Both receptors are involved in induction of cytokine release and CD3 mAb-mediated T-cell activation (8–10). FcγRIIa interacts with $mIgG_1$ and $mIgG_2b$ with low affinity. FcγRIIa, furthermore, has been shown polymorphic in its interaction with mouse IgG_1 antibodies; the FcγRIIa-R131, with Arginine at position 131 was previously named 'high responder' because of its high reactivity with $mIgG_1$, in contrast to the FcγRIIa-H131 (with Histidine at position 131 and was previously named 'low responder'). Binding of $mIgG_2b$ was only poorly to both FcγRIIa allotypes (11,12). We reported earlier that FcγRIIa is effective in ADCC with $mIgG_1$ mAb, but does not perform such activity via $mIgG_2b$ mAb or hybrid $mIgG_2b$–$mIgG_1$ mAb (13). The FcγRIa binds not only conventional monomeric $mIgG_2a$ mAb and $mIgG_3$ mAb (11), but also hybrid

$mIgG_2a$–$mIgG_1$ and $mIgG_2a$–$mIgG_2b$ efficiently (14, 15), which led to the conclusion that $mIgG_2a$ interacts via a single H chain with FcγRIa. Therefore the BsAb, subject of this study, were expected to behave differently in Fc-dependent processes.

Here, we describe the purification and testing of these two different CD3 × CD19 BsAb. Using mouse fibroblasts transfected with the different human Fcγ receptors we show that $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 has the capacity to activate T cells, in contrast to $mIgG_1$–$mIgG_2b$ BsAb/QAI-3. BsAb $mIgG_1$–$mIgG_2b$ (QAI-3) also appeared unable to activate T cells in the presence of freshly isolated peripheral blood monocytes, whereas the $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 could induce T-cell activation in this assay. These CD3 × CD19 BsAb therefore seem suitable reagents to ascertain the role of Fcγ receptor interaction with BsAb in immunotherapy.

Methods

Hybridomas

Monoclonal antibodies and bispecific antibodies used in this study are summarized in Table 1. The hybridomas secreting CD19 mAb (CLB-CD19, of $mIgG_1$ or $mIgG_2a$ isotype) (16,17) or CD3 mAb (CLB-T3/4, $mIgG_1$) (10) were a generous gift of Dr L. A. Aarden (Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam). Isolation of the switch variant CLB-CD19 $mIgG_2a$ hybridoma was previously described (18); applying the same procedure we isolated a $mIgG_2b$ switch variant.

All cell lines were mycoplasma free as determined by a commercial mycoplasma detection system (Gen-Probe, San Diego, CA).

Production of hybrid hybridomas and detection of BsAb

The method to construct hybrid hybridomas was described previously (19). Briefly, we obtained a HAT^s/neomycin^r CD3 hybridoma by isolation of an 8-azaguanine^r (20 μg mL^{-1}; Sigma St. Louis, MO) clone, followed by subsequent retroviral transduction to neo^r (20,21). This cell line was then fused with the wild type $mIgG_2a$ or $mIgG_2b$ isotypic CD19 hybridoma in 50% PEG-4000 (Serva, Heidelberg, Germany) (22). Somatic hybrids were isolated by culturing the fusion mixture in HAT medium (23,24), supplemented with 1 mg mL^{-1} G418 (Gibco, Grand Island, NY). Production of hybrid Ig molecules (different H chains in one Ig) by individual clones was tested by a combination of three assays: (i) presence of two different isotypes in the supernatant from one well in single isotype ELISA (SIE), (ii) by screening supernatants for the presence of two different isotypes within the same Ig molecule (double isotype ELISA, DIE; the SIE and the DIE were performed as previously described (14,21). Briefly, 96-well vinyl plates (Costar, Cambridge, UK) were coated with polyclonal goat-anti-mouse IgG_2a or goat anti-mouse IgG_2b (Southern Biotechnology Associates, Inc.) solutions in PBS for 18 h at 4 °C. After washing three times with PBS, 1% FCS, and 0.1% TWEEN 20, the ELISA plates were incubated with serial dilutions of hybrid hybridoma supernatant or isolated Ab for 2 h at 37 °C. Then, after washing three times, the ELISA plates were incubated with polyclonal $GAMIgG_1$ conjugated to alkaline phosphatase (SBA) for 1 h at 37 °C. Finally, substrate was added to react (PNP, p-nitrophenylphosphatte, Sigma)). (3) presence of isotypically different Ig molecules in the cytoplasm of individual cells by staining ethanol/glacial acetic acid (95/5 (v/v), –20 °C for 15 (min) fixed cells with FITC conjugated subclass specific Ab. Cells were examined by phase contrast immunofluorescence microscopy. After subcloning three to four times, clones were considered stable when constant Ig production was found for at least two months.

Table 1 Parental and bispecific mAb

Parental Ab	Specificity	Isotype	Reference
CLB-T3/4.1	CD3	mouse IgG_1	(10)
CLB-T3/4.2b	CD3	mouse IgG_2b	(10)
WT32	CD3	mouse IgG_2a	(43)
CLB-CD19	CD19	mouse IgG_2a	(16)
CLB-CD19	CD19	mouse IgG_2b	

BsAb	Specificity (isotype)	Reference
QAI-2	CLB-T3/4($mIgG_1$) × CLB-CD19(mIG2a)	
QAI-3	CLB-T3/4($mIgG_1$) × CLB-CD19(mIG2b)	
SHR-1	SHL45.6($rIgG_2b$) × MG1CD19($mIgG_1$)	(34)

Purification of monoclonal- and bispecific- antibodies

(a) *Protein A purification of parental monoclonal antibodies.* Ig present in ascites was isolated using protein A chromatography (protein A Sepharose column, Pharmacia, Uppsala, Sweden) as described (14,19).

(b) *BsAb enrichment using an HPLC-ABx column.* One volume of ascites was diluted with 9 vol. of 25-mM MES buffer with pH5.4 (2-(*N*-morpholino)ethane sulfonic acid (Sigma Chemical Co.). Following injection onto a 7.75-mm × 10-mm Bakerbond ABx HPLC column (J. T. Baker Inc., Chemical Company, Philipsburg, NJ) (25), unbound protein was washed away and bound proteins eluted applying a linear gradient ranging from 0 to 100% (1-M NaAc, pH 7.0) in 90 min. Fractions were collected every 3 min and tested for activity in the DIE and SIE. Active fractions were pooled and dialysed against PBS. Protein concentrations were determined by

the method of Lowry *et al.* (26) using human serum albumin as a standard.

Gel electrophoresis

For IEF, Ig samples were run on 5% polyacrylamide gels under nonreducing conditions with a gradient ranging from pH 3.0 to 10 (Serva, Heidelberg, Germany), as described by the manufacturer. Samples were applied in 15 μL with 2% Ampholines (pH 3–10; Pharmacia). Proteins were fixed using 20% trichloroacetic acid for 10 min and bands visualized with Coomassie blue (Serva) (19). For SDS polyacrylamide gel electrophoresis, Ig fractions were run in a 10–20% gel under reducing conditions(19,27). Proteins were visualized by silver-staining ($AgNO_3$; Merck, Darmstadt, Germany) as described (28).

Immunofluorescence studies

All Ab incubations and wash steps were performed at 4 °C in phosphate-buffered saline containing 1% BSA and 0.1% NaN_3. Fluorescence was quantified using a FACScan (Becton Dickinson, San Jose, CA). Binding curves of the parental CD19 mAb were determined by incubating 10^6 cells with graded amounts of Ab for 30 min. Hereafter, cells were washed once and stained with a FITC conjugated anti-kappa light chain for another 30 min.

Cell lines

The CD19 positive EBV-B cell line APD (29,30), was used as target cell in the cytotoxicity assays with CTL-D11(TCRαβ) as effector cell (29–31). In the immunofluorescence studies the CD19 positive CRL1484(HS-Sultan) plasmacytoma cell line (ATCC, Rockville, MD), and the CD3 positive T-cell line Jurkat (ATCC) were used. The APD cell line, the B cell lymphoma line Raji (ATCC, Rockville, MD), the HLA-1 negative cell line Daudi (ATCC), the CD32 negative cell line MTLM-4 (32) and the pre-pre-B ALL cell line REH (33) were used in the T-cell proliferation assay.

Antibodies

Leu4 (CD3 mAb) and CD45-FITC/CD14-PE (HIe-1/LeuM3) were purchased from Becton Dickinson. The CD28 mAb 15E8 ($mIgG_1$-κ) was kindly provided by Dr R. van Lier (CLB). Antibodies to the kappa light chain and $mIgG_1$, $mIgG_2a$, and $mIgG_2b$ were purchased from SBA (Birmingham, AL). Streptavidin-PE or -FITC were obtained from Becton Dickinson. The CD3 × CD19 $ratIgG_2b$–$mIgG_1$ BsAb/SHR-1 (34), was kindly provided by Dr M. Clark (Cambridge, UK).

Cytotoxicity assay

Cytotoxicity was measured in a conventional chromium release assay in U-bottomed 96-well plates (Nunc, Roskilde, Denmark) using RPMI 1640 medium supplemented with 10-mM HEPES (Gibco), 2-mM L-glutamine, 100 U mL^{-1} penicillin and 100 μg mL^{-1} streptomycin (Gibco), and 10% FCS (Gibco) (31). Briefly, 100 μL of effector cells (CTL-D11/TCRαβ) were mixed with 50 μL of serial dilutions of purified monospecific mAb, purified BsAb, or culture supernatants from hybrid hybridoma clones at a final dilution of 1:4. After 30 min, 2×10^3 ^{51}Cr-labelled target cells (in 50 μL) were added to each well. Plates were incubated at 37 °C for 3 h to allow lysis. After centrifugation, 100 μL of the supernatants were removed from each well to assay the release of ^{51}Cr. Spontaneous release was measured by culturing the target cells in complete medium. Maximum release was determined by incubating the cells in 1% Triton- × 100. Percentage specific release was calculated as 100 × (experimental release – spontaneous release)/(maximum release – spontaneous release). All tests were performed in triplicate.

T-cell proliferation with FcγR transfectants as accessory cells

PBMC were isolated using Ficoll–Paque gradient centrifugation. Monocytes were removed by adherence to plastic. T cells were thereafter isolated by rosetting with 2-aminoethyl-isothiouronium bromide (AET; Sigma) - treated sheep red blood cells (SRBC). Immunofluorescence analysis showed these T-cell preparations to contain more than 96% $CD3^+$ cells, and no $CD14^+$ cells. For T-cell stimulation, flat-bottomed 96-well microculture plates (Nunc) were seeded with irradiated fibroblasts (5000 rad, 10 000 cells $well^{-1}$) which were allowed to adhere for 2 h. CD28 mAb (1 μg mL^{-1}), 10 ng mL^{-1} of test Ab and 30 000 purified T cells $well^{-1}$ were successively added. Each well contained a total of 200 μL cell suspension in culture medium supplemented with 5% FCS $well^{-1}$. Each Ab dilution was tested in triplicate. Proliferation of T cells was measured by uptake of [3H]-thymidine. Cells were cultured for 3 days and for an additional 18 h in the presence of 1 μCi [^{3}H]-thymidine $well^{-1}$. Radioactivity was quantified by scintillation counting. Preparation of FcγR transfectants has been described (8). Briefly, 3T3 mouse fibroblasts were transfected with pDOL-(retroviral expression vector)- containing FcγRIcDNAp135. Mouse 3T6 cells were transfected with FcγRIIa–R131 cDNA pCD-16.2 or pcDX containing FcγRIIa-H131 cDNA pPW3. Expression of IgG Fc receptors was detected by anti-FcγRIa mAb 197 (Medarex, Annandale, NJ) or anti-FcγRIIa mAb IV.3 (Medarex). As a control we also performed experiments in which CD28 mAb was tested, which resulted in background proliferations.

PBMC proliferation assay

PBMC from healthy donors were isolated by Ficoll–Paque density centrifugation and cultured in 96-well round-bottomed microtitre plates at 50 000 cells well^{-1} for 4 days. Cells were stimulated with graded amounts of purified Ab. Experiments were carried out in triplicate, and cell proliferation was quantified as described for T cells.

Target cell induced T-cell proliferation assay

Cells from various CD19$^+$ tumour B-cell lines were exposed to 7000 rad prior to use in the proliferation assay. To 96-well round-bottomed microtitre plates were added; 5×10^4 freshly isolated T cells, and 5×10^4 B cells or 2×10^4 purified autologous monocytes. T cells were stimulated with 10 ng mL^{-1} CD3 × CD19 BsAb, or CD3 mAb, in complete medium containing 10% FCS (200 μL well^{-1}). [^{3}H]-thymidine uptake was measured in triplicate samples of a 4-day culture as described.

Results

Binding kinetics of parental CD19 mAb

Figure 1 shows the binding kinetics of the parental CD19 mIgG$_1$ mAb and the mIgG$_2$a and mIgG$_2$b isotypic variants to the B-cell line CRL1484. This indirect immunofluorescence assay with a wide range of concentrations, indicated that switching of parental mIgG$_1$ mAb to mIgG$_2$a or mIgG$_2$b was only of minor influence on their binding kinetics.

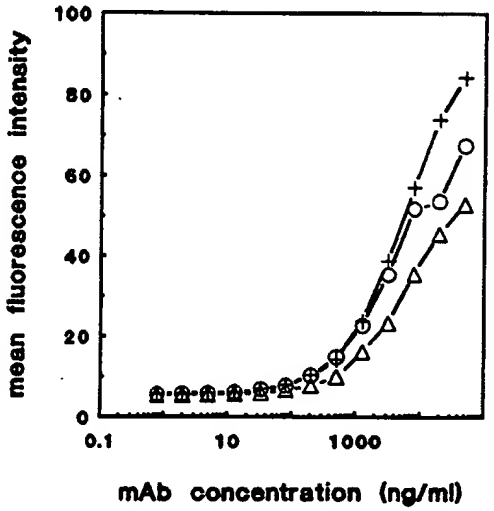

Fig. 1 Binding kinetics of parental CD19 mAb mIgG$_1$ (+) and its two switch-variants mIgG$_2$a (▲), and mIgG$_2$b (○), to the CD19 positive cell line CRL1484 in an indirect immunofluorescence assay with GAM-κ-FITC. Membrane fluorescence is expressed as the mean fluorescence intensity (MFI) on a linear scale (*y*-axis).

Production of hybrid hybridomas

Two hybrid hybridomas were generated by fusion of the parental CD3 mIgG$_1$ hybridoma with the CD19 mIgG$_2$a and the mIgG$_2$b isotypic variants. To isolate somatic hybrids formed between these hybridomas two selection markers were used, both present in the CD3 hybridoma: HAT^r, selected for by culturing in 8-azaguanin, and neomycin^r, introduced by a retroviral vector.

QAI-2: The BsAb producing cell line QAI-2 resulted from fusion of the CD3 mIgG$_1$ hybridoma and the CD19 mIgG$_2$a mAb hybridoma. From a mixture of 4×10^6 parental cells, 10 clones were selected that produced hybrid Ab by the following criteria: First, the DIE performed with supernatant from wells with cell growth showed the presence of both mIgG$_1$ and mIgG$_2$a isotypes within one Ig molecule. Secondly, mIgG$_1$ as well as mIgG$_2$a Ab could be detected by immunofluorescent means in the cytoplasm of individual cells. Hereafter, the presence of functional BsAb in these supernatants was tested by adding them to a mixture of activated T cells and CD19$^+$ B cells (data not shown).

QAI-3: This hybrid cell line was obtained by fusing the CD3 mIgG$_1$ parental hybridoma with the CD19 mIgG$_2$b-producing cell line (fusion efficiency 0.5%). The same procedure was followed as described for QAI-2. The supernatants of clones selected for hybrid Ab production again retargeted the cytolytic activity of T cells towards CD19$^+$ B cells.

From each hybrid hybridoma the clone with the highest capacity to target cytolytic activity was selected for ascites production and subsequent purification.

Purification and biochemical analysis of the hybrid hybridomas

The yield of BsAb in the Ig production of hybrid hybridomas will depend mainly on the extent of homologous vs. heterologous H/L chain pairing. Preferential homologous H/L chain pairing will result in 50% of active BsAb. Preference to associate with the heterologous H chains will result in absence of BsAb, while in case of at random chain pairing approximately 15% of Ig produced constitutes the desired BsAb (19).

To optimize the ion-exchange chromatography procedure, and to determine the localization of parental Ab, also enabling us to detect possible heterologous H/L-chain pairing in our hybrid hybridomas, we started by purifying mixtures of parental Ab. A linear NaAc gradient ranging from 0 to 1 M in 90 min resulted in optimal resolution. Ascites from both hybrid hybridomas were then processed using identical conditions. Figure 2 shows the A_{280} recordings of these experiments. A total of four peaks was resolved. Samples from each peak were analysed in SIE, DIE and for specific binding to CD3 and CD19 antigens. The first peak did not contain Ig based on the results from the ELISA. The next three

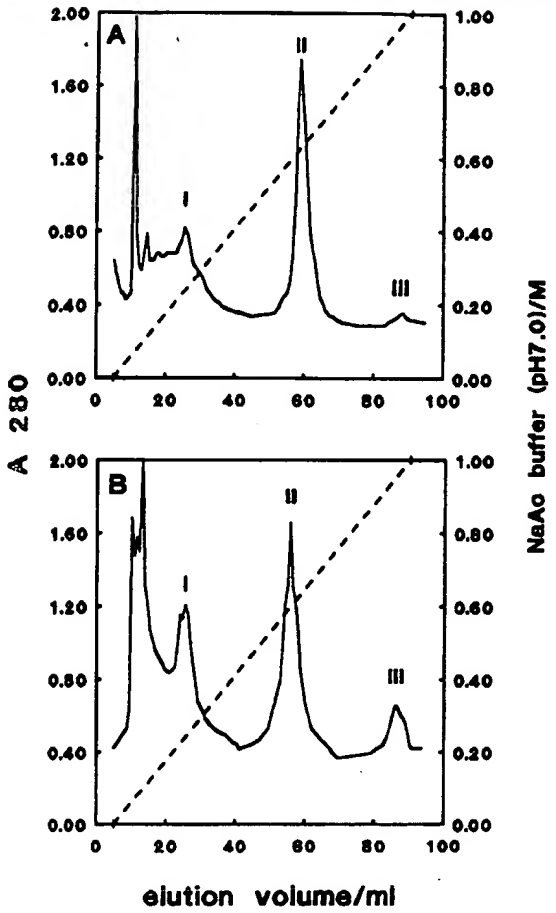

Fig. 2 The elution profiles of the Ig made by the two hybrid hybridomas separated by mixed mode ion-exchange chromatography on a ABx-HPLC column. A_{280} profiles of $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 (A) and $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 (B) are given. BsAb were eluted using a gradient of NaAc ranging from 0 to 1 M (pH 7.0; 1.1% min^{-1}). The elution positions of the BsAbs (peak II), parental CD19 mAb (peak I), and of the parental CD3 mAb (peak III) are indicated.

peaks showed Ab activity (peaks I–III in Fig. 2). Peaks I and III in both the $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 and $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 diagrams coincided with parental CD19, and CD3 mAb, respectively. The presence of hybrid Ab in both the intermediate peaks II, was established by the DIE for $mIgG_1$–$mIgG_2a$ and $mIgG_1$–$mIgG_2b$ Ab.

The production of only three types of Ab, as suggested by the elution profiles showing three distinct and symmetrical peaks, indicated that no heterologous H/L chain pairing occurred in these hybrid hybridomas. The biochemical properties of Ig chains in our hybrid hybridomas did not allow for identification of parental origin. This was true for immuno detection of Western blots obtained from IEF gels run under reducing conditions. Also SDS electrophoresis failed in this respect (data not shown).

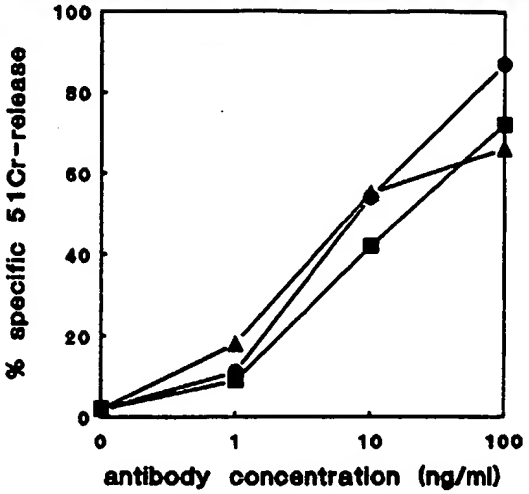

Fig. 3 The capacity of $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 (▲), $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 (■) and $rIgG_2b$–$mIgG_1$ BsAb/SHR-1 (●) to direct T cells towards target cells is illustrated in a cytotoxicity assay. ^{51}Cr-labelled APD cells were added to BsAb preincubated CTL-D11 cells at an E:T ratio of 27:1. ^{51}Cr-release was measured after 3 h incubation at 37 °C and plotted against the concentration of the BsAb.

Bispecific antibody-mediated lysis

The capability of purified QAI-2 and QAI-3 BsAb to mediate lysis was next examined in a ^{51}Cr-release assay and compared to the potency of a previously described CD3 × CD19 BsAb/SHR-1 (31,34). Figure 3 shows these results using the human CTL clone D11(TCRαβ) and the CD19-positive cell line APD. Both $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 and $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 induced lysis of the APD cell line effectively. Even at concentrations of 10 ng mL^{-1} (effector:target ratio of 27:1) significant lysis occurred.

Human T-cell proliferation by BsAb in the presence of FcγR-transfectants: evaluation of binding of QAI-2 and QAI-3 BsAb to Fcγ receptors

To study the ability of QAI-2 and QAI-3 to activate T cells by binding to CD3 and simultaneously interact with FcγR, we measured T-cell activation upon incubation with various Ab and mouse fibroblasts transfected with FcγRIa, FcγRIIa-R131 or FcγRIIa-H131. The results from these experiments are shown in Fig. 4.

The parental $mIgG_1$ CD3 mAb, induced proliferation of T cells in the presence of the FcγRIIa-R131 transfectants, exclusively. To assess the binding of $mIgG_2a$ and $mIgG_2b$ isotypic mAb to these transfectants, as represented by the parental CD19 mAb in $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 and $mIgG_1$–$mIgG_2b$ BsAb/QAI-3, we used a $mIgG_2a$ CD3 mAb (WT32) and the $mIgG_2b$ isotypic variant of the parental CD3 mAb. These Ab did not induce T-cell proliferation in the presence of the

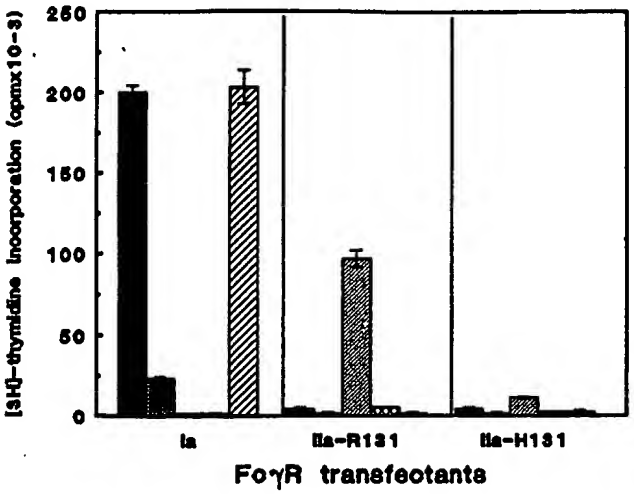

Fig. 4 Human T-cell proliferation induced by cross-linking BsAb and CD3 mAb on human FcγRIa, FcγRIIa-R131 and FcγRIIa-H131 transfectants (see Introduction). Isolated T cells were incubated with an optimal amount (10 ng mL^{-1}) of $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 (■), $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 (▤) or CD3 mAb $mIgG_1$ (▨), $mIgG_2b$ (▩) and $mIgG_2a$/WT32 (▨) in the presence of FcγR-transfected irradiated mouse fibroblasts. Purified CD28 mAb (1 μg mL^{-1}) was added as a costimulatory signal. [^{3}H]-thymidine incorporation was determined on day 4. Results (mean and standard error) are representative for using T cells of four different donors tested in two separate experiments.

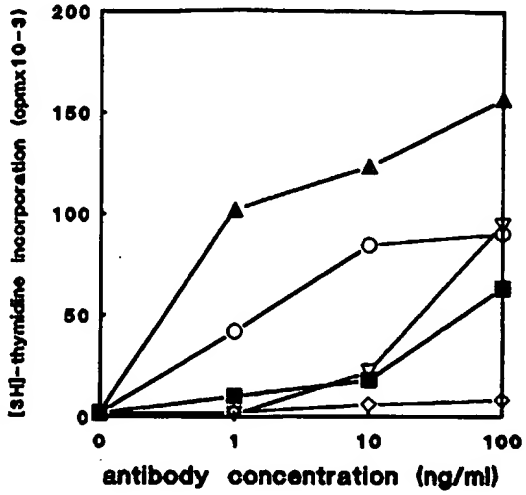

Fig. 5 Proliferation of PBMC induced by graded amount of the $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 (▲), $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 (■) and CD3 mAb $mIgG_1$ (○), $mIgG_2b$ (◇) and $mIgG_2a$/WT32 (▽). The proliferation determined on day four, is shown as the mean of triplicate cultures (variation between triplicates was found < 5%). The experiments were repeated five times, using cells of ten different donors with similar results.

FcγRIIa-H131 transfectant. With the FcγRIa transfectant, T-cell proliferation was found for the $mIgG_2a$ CD3 mAb, but no T-cell proliferation was observed using the $mIgG_2b$ CD3 mAb. The results found with the $mIgG_1$, $mIgG_2a$ and $mIgG_2b$ CD3 mAb agree with the known specificities of these FcγR.

QAI-2, containing H chains of $mIgG_2a$ and $mIgG_1$ isotypes induced T-cell proliferation in the presence of the FcγRIa transfectant only. No induction of T-cell proliferation occurred in the presence of the FcγRIIa-R131 or IIa-H131 transfectants. QAI-3, of which the Fc domain is composed of $mIgG_1$ and $mIgG_2b$, did not induce any T-cell activation. The observations that neither the $mIgG_1$–$mIgG_2a$ BsAb/QAI-2, nor the $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 BsAb interacted with the FcγRIIa-R131 indicated this receptor to be unable to interact with heterodimeric Fc domains in which the $mIgG_1$ isotype is combined with $mIgG_2a$ or $mIgG_2b$. The absence of any T-cell proliferation in these cases, furthermore, excluded the presence of contaminating parental CD3 mAb. The difference in induction of T-cell proliferation by the BsAb was not dependent on the affinity for the CD3 antigen as was confirmed in a competitive binding inhibition assay (data not shown).

Proliferation of PBMC induced by the QAI-2 and QAI-3 BsAb

In order to extend the T-cell proliferation experiments to Ab presenting cells expressing both the FcγRIa as well as the FcγRIIa, PBMC of 10 donors were stimulated with graded quantities of the BsAb and compared to CD3 mAb with different isotypes. Donors were selected for response to $mIgG_1$ CD3 mAb stimulation. A representative experiment is shown in Fig. 5. The proliferation induced by $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 at 1 ng mL^{-1} was 10-fold higher than that by $mIgG_1$–$mIgG_2b$ BsAb/QAI-3. In addition, $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 mediated proliferation was susceptible to an FcγRI-blocking mAb, in contrast to an FcγRII-blocking mAb (n = 3, data not shown). The latter BsAb induced PBMC proliferation only when used in concentrations of 100ng mL^{-1}. All individuals responded to $mIgG_1$ and $mIgG_2a$ (WT32) CD3 mAb stimulation, although stimulation by the $mIgG_2a$ mAb (WT32) was limited at low concentrations. This may be explained by the lower affinity of the WT32 mAb (35). In our donor population no responders to the $mIgG_2b$ CD3 mAb were identified.

Target-cell-induced proliferation of T cells

To investigate whether $CD19^+$ target cells could replace the role of monocytes in inducing T-cell proliferation by CD3 × CD19 BsAb, B-cell lines with different phenotypes were tested. Freshly isolated T cells did not proliferate when incubated with either of the tumour B-cell lines (originating from EBV, Burkitt's lymphoma and

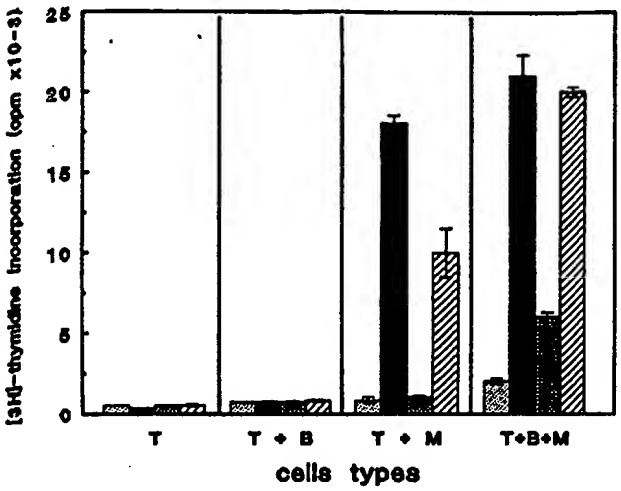

Fig. 6 Accessory cell induced T-cell proliferation in the presence of CD3 mAb or CD3 × CD19 BsAb. Purified T cells (T) were cultured for 4 days with irradiated B cells from the CD19 positive EBV-B cell line, APD (B), at a T:B cell ratio of 1:1, and with autologous monocytes (M) (T:mono ratio of 10:4). Antibodies examined are added directly to the well and include: $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 (■), $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 (▩) and CD3 mAb $mIgG_2a$, (▨). No antibody (□). Results (mean and standard error) represent four different experiments. T-cell proliferation was measured by 18 h uptake of [^{3}H]-thymidine as described.

cALL) and 10 ng mL^{-1} of the BsAb. As shown in Fig. 6, even the EBV-B cell line, APD, could not induce T-cell proliferation in a T:B cell ratio of 1:1 although this cell line was found to express surface molecules like CD11a/LFA-1, CD54/ICAM-1, B7, and CD32 (36). Proliferation was markedly increased when autologous monocytes were added to the wells.

Discussion

Bispecific antibodies can be used to target the cytotoxic capacity of activated T cells to tumour cells. In monospecific mAb therapy, the Fc domain constitutes an essential component in the therapeutic action of the molecule. FcR-mediated cross-linking, however, has been shown to trigger cytokine release and subsequent toxic side effects (2,11,37). *In-vivo* application with biologically produced BsAb has been proposed due to their stability (38). When used in a clinical setting, however, these BsAb are often administered without their Fc part to prevent interaction with FcγR on monocytes and to diminish production of anti-mouse antibodies. Although BsAb can be effective without this domain (39,40), interaction with FcγR may also be beneficial by inducing a target cell-independent induction of a cytotoxic phenotype in the effector cell, especially when no *ex vivo* activated T cells are used. The same interaction may also trigger T-cell proliferation (6,10). After BsAb mediated targeting, the activated cytotoxic T cells may subsequently kill tumour cells.

We constructed two BsAb for immunotherapy of patients with B cell malignancy. The BsAb have identical CD3 and CD19 antigen binding, but differ in their Fc domain composition. From previous studies in ADCC we knew that $mIgG_1$–$mIgG_2a$ hybrid Fc domains bind as effectively to FcγRIa as regular $mIgG_2a$ (13,14). These molecules show minimal interaction with FcγRIIa binding $mIgG_1$ (14). Hybrid $mIgG_1$–$mIgG_2b$ Fc domains do not interact appreciably with FcγRIa and FcγRIIa (14). We anticipated that the differences seen in ADCC with hybrid $mIgG_1$–$mIgG_2a$ and $mIgG_1$–$mIgG_2b$ Fc domains (13,15) would also affect the capacity of such Ab to induce T-cell activation or cytokine release. Previous studies using mouse mAb have reported on the influence of the H chain isotype on the mitogenic properties of CD3 mAb (5,6,10,41) and on the release of IL-6 or TNF-α after cross-linking of FcγR by several mouse isotypes (8,9). This has not been analysed for hybrid (H chain) Ab. Detailed knowledge on the role of hybrid Fc domains in CD3 mediated T-cell activation by therapeutic BsAb, as reported here, may be of predictive value for applications *in vivo*.

The hybrid hybridomas were produced as reported earlier (21), by using PEG-mediated fusion of a HAT^s/neo^r cell line with a 'wild-type' line. Both BsAb, QAI-2 (CLB-T3/4.m1 × CLB-CD19.m2a) and QAI-3 (CLB-T3/4.m1 × CLB-CD19.m2b) were selected by testing the supernatant of producing clones in DIE and cytotoxicity assays. Purification of the BsAb was performed on a HPLC-ABx column. The A_{280} profiles of QAI-2 and QAI-3, showed only three symmetrical peaks with clear intervals, suggesting preferential homologous H- and L-chain pairing.

The role of these purified BsAb in T-cell activation was evaluated. $mIgG_1$-IgG_2a BsAb/QAI-2 did induce T-cell activation when presented by FcγRIa transfectants, extending our previous findings in ADCC experiments (13). Apparently, the presence of only one $mIgG_2a$ H chain in an Fc domain suffices for binding. Also in line with the ADCC results, the $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 did not induce a significant T-cell proliferation after interaction with the FcγRIIa-R131 transfectant. Moreover, the complete absence of T-cell activation with both $mIgG_1$–$mIgG_2a$ BsAb/QAI-2, and $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 using FcγRIIa-transfectants as accessory cells, strongly argues for the absence of parental CD3 $mIgG_1$ mAb in our BsAb preparations, which in turn reinforces the conclusions drawn. BsAb $mIgG_1$–$mIgG_2a$ QAI-2 stimulates T-cell proliferation to the same extent as seen with parental CD3 mAb, despite its monovalency for the CD3 antigen. Therefore, FcγR interaction of this mAb may well serve to activate

the T cell *in vivo* as a preliminary step in targeting cellular cytotoxicity. Although both BsAb bind to CD3 and CD19 antigens, they did not induce T-cell proliferation in the presence of several CD19 positive tumour cell lines, but did so when autologous monocytes were added. The Fc domain thus seemed a necessity for T-cell activation.

In conclusion, stimulation by $mIgG_1$–$mIgG_2a$ BsAb/QAI-2 induces T-cell proliferation when supported by FcγRIa-positive accessory cells. CD19 positive B cells alone could not induce this BsAb mediated T-cell proliferation. The binding of the BsAb to the FcγR may be of great advantage for the induction of T-cell activation, and is independent of the FcRIIa allotype of the donor. However, cross-linking of FcγRIa on monocytes may at the same time trigger them to produce cytokines. These cytokines (i.e. IL-6 and TNF-α) are probably responsible for the severe side-effects seen in mAb treatment (2,3,42). The $mIgG_1$–$mIgG_2b$ BsAb/QAI-3 could not induce T-cell mitogenesis and is therefore most likely not able to bind to the examined FcγR and is thus not expected to induce cytokine release by accessory cells. For effective use of this BsAb additional signalling by IL-2 might be needed. Only *in vivo* experiments will show whether these assumptions prove to be correct.

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